Daily consumption of aronia berry (poly)phenols improves endothelial function and modulates the gut microbiome in healthy volunteers: a double-blind randomized controlled trial

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Short running head: Aronia Berry Consumption on Vascular Function.

Abbreviations: Randomized Controlled Trial, RCT; Augmentation Index, AI; cardiovascular disease, CVD; coronary artery disease, CAD; coronary heart disease, CHD; flow-mediated dilation, FMD; pulse wave analysis, PWA; pulse wave velocity, PWV; randomized controlled trial, RCT; total (poly)phenols, TP; anthocyanins, ACN.

Clinical trial registry: The National Institutes of Health (NIH)-randomized trial records held on the NIH ClinicalTrials.gov website (NCT03041961). *Aronia Berry Consumption on Vascular Function.*

Abstract

Objective: *Aronia melanocarpa* is a rich source of polyphenolic compounds. Most studies investigating the cardiovascular health benefits of berries have been conducted in individuals at high risk or with cardiovascular disease using relatively high amounts of fruit or juice that are impractical for daily consumption. This study aims to investigate the effect of dietary achievable amounts of aronia berries on vascular function and their impact on the faecal microbiota composition in a young and healthy population.

Methods: A double-blind, placebo-controlled, parallel designed study was conducted in 66 healthy male adults randomly allocated to a polyphenol rich extract (40% total (poly)phenols, equivalent to 75 g of aronia berries), a whole fruit powder (4% total (poly)phenols, equivalent to 10 g of aronia berries) or placebo (artificially coloured maltodextrin, 0% total (poly)phenols). Each participant ingested one 500 mg capsule every day for 12 weeks. Flow-mediated dilation (FMD), pulse wave velocity, augmentation index, blood pressure, heart rate and serum biochemistry were assessed at baseline and after 12 weeks. FMD was also measured 2 h after capsule intake on both visits. Plasma (poly)phenol metabolites were analysed using micro-elution solid phase-extraction coupled with LC-MS. Gut microbiota composition was determined via 16S rRNA Sequencing in stool samples collected at baseline and at 12 weeks.

Results: Consumption of aronia whole fruit and aronia extract powder for 12 weeks led to a significant increase in FMD over control of 0.9 ± 0.4 % (95 % CI: 0.13 to 1.72) and 1.2 ± 0.4 % (95 % CI: 0.36 to 1.97) respectively. Acute improvements in FMD were also observed 2h after consumption of aronia extract on day 1 (1.1 ± 0.3,

p=0.003) and 12 weeks later (1.5 ± 0.4 %, p=0.0001). A parallel increase in circulating plasma phenolic metabolites was found 2 h post-consumption and after 12 week daily consumption of the aronia treatments even after an overnight fast. No significant differences in arterial stiffness, blood pressure or blood lipids were found. A correlations analysis identified potential associations between aronia-derived phenolic metabolites and specific gut microbial genera.

Conclusions: In healthy young men, acute and daily consumption of aronia berry (poly)phenols improved endothelial function and modulated gut microbiota composition indicating that regular aronia consumption has the potential to maintain cardiovascular health in healthy individuals at low risk of cardiovascular disease.

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1. Introduction

Diet is one of the most important lifestyle factors greatly influencing the incidence and progression of cardiovascular diseases (CVD) (1). Convincing evidence from epidemiological studies suggests that high intakes of fruit and vegetables reduces the risk of developing coronary heart disease and stroke (2-4). This may be, in part, thanks to the high amounts of bioactive compounds present in fruits and vegetables (5). More evidence from human intervention studies suggest that daily consumption of foods high in (poly)phenols, such as tea, cocoa and berries, may improve long term vascular health (6). A limited number of randomized controlled trials (RCTs) have demonstrated small improvements in blood lipids, blood pressure and endothelial function following daily consumption of berries or other anthocyanin rich foods (7-9). However, due to the low number of RCTs and large heterogeneity between study designs, study populations and interventions used, there are some inconsistencies in findings, as decribed by one meta-analysis on anthocyanin-rich foods (10).

The gut microbiome has an important influence on human health and a limited amount of evidence indicates that gut microbes might be modulated upon consumption of dietary (poly)phenols by humans. In a study by Queipo-Ortuño *et al.,* intake of red wine by healthy volunteers over 4 weeks was parralelled with a significant increase of several microbial communities including *Bifidobacterium, Bacteroides* and *Enterococcus* (11). Even more specifically, anthocyanin microbial metabolites from red wine were previously associated with higher levels of *Bifidobacterium,* which has been associated with beneficial health effects (12, 13). Similarly, in a study with healthy humans, significant increases in *Bifidobacterium* was observed 6-weeks after consumption of an anthocyanin-rich wild blueberry powder (14).

One berry of growing interest is *Aronia melanocarpa*, as it has one of the highest (poly)phenol content compared with other fruits (15, 16). Few RCT's have shown that daily aronia berry consumption decreased blood pressure in individuals with cardiovascular disease (17), metabolic syndrome (18) or at high risk of CVD (17, 19). Whether or not regular aronia consumption has the potential to maintain and/or improve cardiovascular health in individuals without CVD and those with low degrees of cardiovascular risk, that is, healthy subjects, has not been evaluated. In addition, the effects of aronia berries on FMD have not been investigated before in a RCT. The primary aim of the present study was to investigate the effects of acute and daily consumption of dietary achievable amounts of aronia whole berries and a (poly)phenol-rich extract on endothelial function in a population of young individuals at low risk of CVD. Finally, to explore the role of aronia intake on the gut microbial ecology, characterization and profiling of gut microbiota was assessed as a secondary outcome.

2. Methods

2.1. Intervention Study Subjects

Sixty-six healthy male volunteers aged 18-45 years were recruited from King's College London and the surrounding area. Health was ascertained by a routine clinical physical examination and specific medical history questionnaire. Volunteers with manifest cardiovascular disease including coronary artery disease, cerebrovascular disease and peripheral artery disease were excluded (Figure 1). Additional exclusions were: hypertension (≥140 mmHg SBP and ≥90 mmHg DBP), body mass index ≥30kg/m^2, diabetes mellitus and metabolic syndrome, acute inflammation, terminal renal failure, malignancies and abnormal heart rhythm (<60 or >100 bpm). Subjects were also excluded if they had allergies to berries or other significant foods, were using medication or other dietary supplements, smoked an irregular number of cigarettes per day or were planning to quit in the next 6 months.

2.2. Study Design

A three-arm, double-blind, parallel, randomized controlled trial was conducted. Informed consent was obtained and subjects were randomized to the treatments. We investigated the effects of two formulations of aronia berry capsules on vascular function compared with a placebo control capsule. Measurements were taken at baseline and 2 h post-acute consumption, and this was repeated 12 weeks after daily capsule consumption.

Once participants were screened and included, they attended two visits, at baseline and 12 weeks later. Participants were instructed not to alter their usual dietary habits nor their physical activity throughout the study. Participants were asked to refrain from eating high (poly)phenol products 24 h prior to the first visit, such as red wine and

beer, fruits and vegetables, nuts, olive oil, coffee, chocolate or cocoa products and tea. Dietary recalls (24 h) were completed at the start of each visit to monitor the low (poly)phenol diet. Food diaries and physical activity questionnaires were completed before and during the trial to ensure their habitual dietary and fluid intake remained consistent. Additionally, participants were fasted 12 h prior to each visit. Measurements of FMD, peripheral BP, PWV, AIx as well as blood samples were all taken at baseline (0 h) then again 2 h post-acute consumption of one capsule (Figure 2). Stool samples were collected by the participants, using Omnigene-gut stool kits (DNA Genotek), and stored at -20°C at the beginning of each visit. Participants were followed up three times throughout the 12-week period via phone to ensure no adverse events occurred.

The study investigated both the acute (0-2 h) and chronic (0-12 weeks) effects of aronia berry (poly)phenols. The primary endpoint was endothelial function measured by flow-mediated dilation (FMD) using high-resolution ultrasound. Secondary endpoints included; pulse-wave velocity (PWV), augmentation index (AIx) and blood pressure (ambulatory and central) as determined automatically by a blood pressure monitoring system and applanation tonometry (Sphygmocor). Tertiary outcomes were plasma glucose, lipids, phenolic metabolites and gut microbiota.

A team of qualified researchers enrolled participants on the study and assigned the interventions. Participants and researchers administering interventions and assessing outcomes were blinded to the intervention groups. An independent researcher generated the random allocation to treatment sequence using a random number generator with the purpose to allocate a specific number to every volunteer. This way, information about group allocation remained concealed. When study visits and analysis of primary outcome were completed, the independent researcher provided

the codes for unblinding and treatment grouping. The study was conducted in accordance to the guidelines stated in the current revision of the Declaration of Helsinki. All procedures were approved by King's College London Ethics Committee (HR-15/16-3739; Registration number: NCT03041961). Volunteers were assessed and data were collected in detailed case report forms between February and July 2017 in the Metabolic Research Unit at the Department of Nutritional Sciences of King's College London.

2.3. Aronia berry and control capsules

Capsules were provided and manufactured by Naturex-DBS, LLC (Sagamore, Massachusetts, USA). The "aronia extract" capsules were concentrated extracts rich in (poly)phenols and processed by the removal of fibre and organic acids from 75 g aronia berries, containing 115 mg total (poly)phenols (**Table 1**). The "aronia whole fruit" capsules contained the equivalent to 10 g of the whole aronia berry fruit, and containing 20 mg of total (poly)phenols (**Table 1**). The control capsules, matched in appearance to both treatment capsules, contained maltodextrin and no (poly)phenols. The capsules were all matched in weight, carbohydrates and calories (**Supplementary Table 1**). Capsules were stored in plastic bottles with patient randomization number and unique treatment allocation number.

2.4. Biochemical analyses

Blood samples collected in EDTA/heparin tubes (Greiner Bio-One Ltd., Gloucestershire, UK) were centrifuged (1700 *g*; 15 min; 4˚C) immediately after collection. Plasma samples for (poly)phenol analysis were spiked with 2% formic acid and frozen at -80°C. All clinical chemistry parameters including total cholesterol, LDL

and HDL-cholesterol, TAG (enzymatic photometric assay; RocheDiagnostics), glucose, Hb_{A1c} and whole blood count were analysed according to standard procedures (Biochemistry department, King's College Hospital, Denmark Hill, London).

2.5. Dietary assessment of background diet

To assess dietary intake, 7-day food diaries by EPIC (European Prospective Investigation of Cancer; University of Cambridge) were completed by participants before the first visit (baseline) and at 11 weeks, before attending the second visit, to ensure habitual diets did not change during the study period. Participants were instructed to provide as much detail as possible about all food and drinks consumed. Average daily macro- and micronutrient composition of participant's diet was analysed with the use of Nutritics (Nutritics Professional Diet Analysis, version 3.74; Nutritics Ltd). Polyphenol intakes were assessed using the online free database Phenol-Explorer (http://phenol-explorer.eu).

2.6. Vascular measurements

FMD of the brachial artery was measured as previously described (4). Briefly, the diameter and flow velocity of the brachial artery was measured using a 12 MHz transducer (Vivid I, GE healthcare, Buckinghamshire, UK) and automatic edge-detection software (Brachial Analyser, Medical imaging applications, Iowa City, USA) yielding standard deviations of mean differences between repeated measurements of less than 1%. Brachial artery diameter was measured 2 cm proximal to the elbow. Reactive hyperaemia was induced by 5 min of distal lower arm occlusion with sphygmomanometric cuff inflated to 180 mm Hg. Blood flow was recorded at baseline

using the Doppler mode. A forearm blood-pressure cuff was placed distal to the antecubital fossa and inflated to 180 mmHg for 5 min. Diameter was measured at baseline and immediately after cuff deflation at 20, 40, 60 and 80 sec, the diameter was assessed and FMD calculated as maximal relative diameter gain relative to baseline. The FMD was expressed as $(diameter_{max}\text{-}diameter_{baseline})/diameter_{baseline}$ x100.

Central BP parameters including AIx were measured by applanation tonometry using the SphygmoCor® (SMART medical, Gloucestershire, UK). Via a transfer function, the pressure waveform of the ascending aorta was synthesized. PWV was determined using measurements taken at the carotid and femoral artery as previously described (20).

2.7. *UHPLC Orbitrap MS analysis of plasma (poly)phenols*

Sample preparation and solid phase extraction for plasma (poly)phenol analysis was performed as described previously (21). The detection of plasma (poly)phenol metabolites was performed on a Exactive^{TM} Orbitrap Mass Spectrometer (Thermo Scientific, CA, USA) after separation on a Accela 1250 pump UHPLC system (Thermo Scientific, CA, USA). The autosampler injected of 5 µL of each sample in a Zorbax Eclipse Plus RRHD column 2.1 × 50 mm, 1.8 m with a compatible Eclipse Plus guardcolumn 2.1 × 5 mm, 1.8 m (Agilent, Waldbronn, Germany). The mobile phase consisted of 0.1% HCOOH (solvent A) and acetonitrile with 0.1% HCOOH (solvent B) in a 10 min gradient program. Quantification analysis of the plasma (poly)phenols was done using Xcalibur 2.2 (Thermo Scientific, CA, USA).

2.8 Faecal sample collection and microbiome analysis

Faecal samples were collected in the week before each study visit using OMNIgene®•GUT self-collection tubes (DNA Genotek, Ottawa, Canada) and were stored in -20°C until further analysis. Microbiome analysis was performed by Clinical-Microbiomics A/S (Copenhagen, Denmark) as described elsewhere (22). Briefly, Total microbial DNA was extracted from faeces using the 96-well NucleoSpin Soil DNA Isolation Kit (Macherey-Nagel, Düren, Germany). PCR was performed with 16S rDNA primers S-D-Bact-0341-b-S-17 and reverse primer S-D-Bact-0785-a-A-21 with Illumina adapters attached (23) in order to target the V3-V4 regions. The following PCR program was used: 98 °C for 30 sec, 25x (98° C for 10 s, 55 °C for 20 s, 72 °C for 20 s), 72 °C for 5 min. Sequencing was performed on an Illumina MiSeq desktop sequencer using the MiSeq Reagent Kit V2 (Illumina, San Diego) for 2x250 bp paired-end sequencing.

The 64-bit version of USEARCH (24) and mothur (25) were used in combination with several in-house programs for bioinformatics analysis of the sequence data. Following tag identification and trimming, all sequences from all samples were pooled. Sequences were clustered at 97 % sequence similarity. Additional suspected chimeric OTUs were discarded based on comparison with the Ribosomal Database Project classifier training set v9 (26) using UCHIME (27). Taxonomic assignment of OTUs was done using the method by Wang et al. (28) using the database from the Ribosomal Database Project. To find modifications in the microbiota structure associated with the aronia treatments, the samples were also longitudinally analysed by RandomForest. Briefly, Random Forest is a powerful classifier that identifies the best subset of features (here, relative genus abundance) that can discriminate between categories (time points). In particular, we applied the algorytm to the three groups separetely

(aronia extract, aronia whole fruit and control). The significance in the abundance of the relevant taxa were validated by Wilcoxon signed-rank tests.

2.9. Power calculation and statistical analysis

Power calculations were performed for the primary end point: change in FMD response after consumption. The power was based on the inter-individual variability for FMD measurement of the operator (SD = 1%). Assuming an 80% power, and a 0.05 significance level, the total number subject required to provide sufficient power to detect a 1% difference change in FMD in a three-arm parallel study is 60 (n=20 per arm). Assuming a 10% drop out, 22 participants per arm should be recruited. Changes in FMD (%) between control and treatment groups were performed using a paired one-way ANOVA with Tukey's post-hoc test. Paired sample t-tests were performed on dietary assessment data to test for any significant differences. One-way ANOVA were performed on baseline dietary assessment data to ensure there were no differences in polyphenol, micro- or macronutrient intakes. Correlations are presented as Pearson's r for non-normal distribution and as Spearman for normal distribution. Statistical analysis were performed with the use of IBM SPSS Statistics 22.0 (Statistical Product and Service Solutions; IBM Corp) and GraphPad Prism version 7 for Windows, (GraphPad Software, La Jolla California USA). Statistical significance is accepted at $p<0.05$.

3. Results

3.1. Baseline characteristics of the study population

A total of 84 volunteers were considered for participation in the study, of which 18 were excluded and 66 were included and randomised into the three intervention groups (**Figure 1**). The first study visit started in February and the last study visit ended in July 2017. A total of 3 follow-up calls were performed per participant between study visits. Two participants discontinued the study after the first visit, and 64 completed both visits (drop-out rate of 3%). The baseline characteristics of the different groups of healthy young men were all within the normal range and no differences were found between treatment groups (**Table 2**).

3.2. Safety and tolerance of the interventions

All the treatments were well tolerated and only 4 potential adverse events were reported over the course of the study, and were considered unrelated to the treatments: 2 of the participants in the aronia extract group reported unusual tiredness, 1 volunteer in the aronia whole fruit group reported a persistent cough for a few weeks and in the control group, 1 volunteer reported food poisoning. **Supplementary table 2** shows the 10 safety parameters assessed, which remained in the normal healthy range after 12 weeks of treatment.

3.4. Dietary assessment of food diaries and evaluation of background diet

Analysis of the 7-day food diaries of study participants revealed no significant differences in micro- and macronutrient intakes as well as polyphenol intake between any treatment group prior to the start of the study (**Supplementary Table 3**). At baseline participants had an average daily (poly)phenol intake of 531 ± 357 mg, of which 23 ± 8 mg of that being anthocyanins (**Supplementary Table 3**).

3.3. Efficacy of aronia interventions on vascular function

Our primary outcome was changes in FMD after 12 weeks daily supplementation. Repetitive intake of aronia extract and aronia whole fruit significantly improved FMD by 1.0 ± 0.2 % and 0.8 ± 0.3 % at baseline of week 12 in comparison to day 1, respectively. When compared to control treatment, changes in FMD after aronia extract and aronia whole fruit consumption were significantly higher by 1.2 % (95 % CI: 0.36 to 1.97) and 0.9 % (95 % CI: 0.13 to 1.72), respectively (**Figure 2, Supplementary Table 4**).

Acute improvements in FMD were also investigated with a significant increase in FMD of 1.4 ± 0.2 % and 1.5 ± 0.2 % observed after 2 h consumption of aronia extract on day 1 and week 12 in comparison with baseline, respectively (**Figure 2**, **Supplementary Table 4**). In comparison with control, aronia extract increased significantly by 1.1 % (95 % CI: 0.37 to 1.78) and 1.7 % (95 % CI: 0.62 to 2.34) after 2 h on day 1 and week 12, respectively. No significant acute FMD changes with respect to baseline or control were observed for the aronia whole fruit group (**Figure 2**, **Supplementary Table 4**).

No significant differences in the secondary outcomes including peripheral and central blood pressure, arterial stiffness and blood lipids were observed in any of the treatment groups (**Supplementary Tables 4 and 5**).

3.5 Phenolic metabolites increase in plasma after aronia consumption

Detailed targeted metabolomics analysis of plasma samples was performed and 63 phenolic metabolites were quantified at baseline and after consumption of all capsules, including derivatives of hippuric acids, benzoic acids, hydroxycinnamic acids, phenylacetic acids, propionic acids, benzaldehydes, catechols, pyrogallols, flavonols

and valerolactones (**Supplementary figure 1**). Most metabolites were present in nanomolar concentrations, except for hippuric acid, benzoic acid, phenylacetic acid and 3-(4-hydroxyphenyl)propionic acid, which were present at micromolar levels even at baseline. The highest total (poly)phenol concentrations were found 2 h upon extract consumption and mounted up to 301 ± 239 µM. The extract group showed increases in total (poly)phenol concentrations of 166 ± 171 µM and 30 ± 156 µM after 2 h and 12 weeks respectively. The whole fruit group showed substantially lower plasma total (poly)phenol increases of 43 ± 125 µM and 14 ± 106 µM after 2 h and 12 weeks of consumption respectively. No significant differences in total plasma (poly)phenols were found between the three intervention groups at baseline, with the exception of phenylacetic acid ($p=0.01$), 2-hydroxybenzoic acid ($p<0.001$), homovanillic acid ($p=0.048$) and homovanillic acid sulfate ($p=0.04$), which were significantly higher in the aronia whole fruit group (data not shown).

At 2 h postconsumption, 48 compounds increased significantly with respect to baseline in the aronia extract group (19 hydroxycinnamic acid derivatives, 13 benzoic acids, 5 flavonols, 4 phenylacetic acids, 2 propionic acids, 2 benzaldehydes, 1 hippuric, 1 pyrogallol and 1 valerolactone), while 22 compounds increased significantly after consumption of the aronia whole fruit (9 benzoic acids, 4 hydroxycinnamic acids, 3 phenylacetic acids, 2 flavonols, 2 benzaldehydes, 1 hippuric and 1 pyrogallol derivative). Only one compound, 1-Methylpyrogallol-*O*-sulfate, increased significantly after consumption of the control capsule.

Repetitive intake of the capsules for 12 weeks led to significantly increased baseline plasma levels of 18 compounds in the aronia extract group, 10 compounds in the whole berry group and 4 compounds in the control group. The chronic changes

observed in the treatment groups were predominantly driven by phenylacetic acids, benzoic acids, hydroxycinnamic acids, flavonols and benzaldehydes (**Figure 3**).

On week 12, plasma phenolic metabolites also increased significantly 2 h post-consumption of aronia extract (2 hippuric acids, 5 benzoic acids, 7 hydroxycinnamic acid derivatives, 2 phenylacetic acids, 2 benzaldehydes, 2 flavonols, 1 propionic acid and 1 valerolactone) and aronia whole fruit (2 hippuric acids, 5 benzoic acids, 6 hydroxycinnamic acid derivatives, 2 benzaldehydes, 1 phenylacetic acid, 1 propionic acid, 1 catechol derivative and 1 flavonol), while only 1 compound (phenylacetic acid) increased in the control group.

Correlation analysis between changes in plasma metabolites and changes in flow-mediated dilation with respect to baseline revealed significant correlations in 20 metabolites after aronia extract consumption and in 5 metabolites after consumption of the whole fruit treatment (**Table 3**).

3.6 Effects on the gut microbiota correlations with plasma (poly)phenol metabolites

Faecal samples were taken on the first and last day of the study to conduct genomic analysis of microbial communities. To test the hyphothesis that aronia supplements can lead the human gut microbiome to different configurations, we first analyzed the overall microbiome diversity - described in terms of the diversity within a sample, (i.e. alpha diversity) and between samples (beta diversity). Microbial diversity was very high and not significant in any of the treatment groups after aronia intake (data not shown). To explore the relationship between (poly)phenol metabolites and gut microbial genera, correlation heatmaps were created (**Figure 4**). The change in a subset of metabolites measured in circulation after 12-week intake of both extract and control capsules were correlated with the corresponding changes in gut microbial

genera abundances (**Figure 4A**). In a similar way, a correlation heatmap was performed using data from whole fruit and control groups (**Figure 4B**). At a first glance, **Figure 4** shows that most of the correlations were not significant in either of the 2 matrices. However, the figure clearly shows that significant correlations were found in both matrices, with more significant associations after intake of the extract compared to whole fruit. Furthermore, Random Forests was applied to predict the bacterial genera that would discriminate between the different treatment groups. Treatment-discriminatory bacterial genera were identified with distinctive modifications in their relative abundances, which were validated by Wilcoxon signed-rank tests with the following results: the aronia extract group had a significant higher abundance of *Anaerostipes* (+10.6%, p=0.01), the whole fruit group showed significant increases in *Bacteroides* (+193 %, p=0.01) and *Clostridium XiVb* was significantly higher (+2.5 %, p=0.01) after placebo treatment (**Supplementary Figure 3**). The difference of the changes in % abundancy were also calculated between treatment groups and revealed significant higher increases in *Anaerostipes* (21 %, p=0.04) when comparing aronia extract group to placebo group (**Supplementary Figure 4**).

4. Discussion

In the present study, we showed for the first time that daily consumption of aronia whole fruit powder as well as a polyphenol-rich aronia berry extract over 12 weeks improved FMD by 0.9 ± 0.4 and 1.2 ± 0.4 %, respectively as compared to control. In addition, acute (2 h) improvements were observed after intake of aronia extract on day 1 (1.1 %; 95 % CI: 0.37 to 1.78) and after 12 weeks (1.5; 95 % CI: 0.62 to 2.34) with respect to control. The effects were paralleled with a baseline increase in plasma phenolic metabolites and with significant changes in the gut microbiota populations. Safety parameters for capsule intake were measured and based on the results in **Supplementary Table 2**, the capsules were considered safe with no associated adverse events. Intake of the aronia extract and whole fruit capsules significantly improved the first outcome - FMD - for 1.0 ± 0.2 % ($p = 0.0006$) and 0.8 ± 0.3 % ($p = 0.049$) after 12 weeks supplementation. Being a validated prognostic marker for cardiovascular risk (29), the predictive FMD strength towards CVD risk was previously analyzed in a meta-analysis of 23 RCTs (13422 participants) where improvement in 1 % FMD was associated with a decrease in overall CVD risk of 8 to 10 % over 4 years (30). It came to our attention that very few studies reported FMD improvements after repetitive intake (poly)phenol-rich foods in healthy people (5, 31-34). In one study, by Khan *et al.*, a blackcurrant juice (204 mg TP) improved baseline FMD significantly by 1.1 % in healthy people after 6 weeks of daily intake (34). The anthocyanin dose given by Khan *et al.* (36 mg) was very similar than the one present in the aronia extract capsules from this study (29 mg) and could explain why improvements in FMD are comparable with the one found in our study (1.1 % by Khan *et al.* vs. 1.0 % in current study), indicating that daily achievable doses of aronia might be effective in improving and maintaining vascular function over a longer period. The applicability of our findings

382 is underlined by an updated publication of EFSA requirements for FMD-based health
383 claims related to cardiovascular health (35). In January 2018, EFSA stated that a
384 sustained (at least 4 weeks) increase in FMD after an overnight fast in response to a
385 food intervention is regarded as a beneficial physiological effect. As compared to the
386 1.0 % improvements in FMD observed after extract consumption, slightly lower FMD
387 enhancement (0.68 %, 95% CI 0.46–0.90) were found after statin therapy in a meta-
388 analysis of 46 RCTs (36), which highlights the clinical relevance of repetitive aronia
389 intake in established primary and secondary prevention applications. Significant acute
390 improvements in FMD were also found 2 hours upon consumption of the aronia extract
391 (1.4 ± 0.2 %, $p < 0.0001$) but not after whole fruit intake (0.4 ± 0.2 %, $p = 0.7$)
392 (**Supplementary table 4**). The low (poly)phenol content in the whole fruit capsules
393 might not have been enough to elicit acute FMD changes, which was partly reflected
394 in the plasma of volunteers 2 h post-consumption where extract consumers showed 4
395 times higher increases in total plasma (poly)phenol concentration than whole fruit
396 consumers. We cannot discard that there may be other components in the whole fruit,
397 such as fibers and vitamins, that may be responsible for the sustained effects in
398 endothelial function observed in this work. Only one other study investigated plasma
399 phenolic acid concentrations after intake of a 500 mg aronia extract capsule with
400 increases in 5 plasma and 8 urine (poly)phenols and identified hippuric acid as major
401 recovered metabolite (37). In our current work, a much more extensive targeted
402 metabolomics approach using 63 authentic (poly)phenol standards - including 23
403 phase II sulfate and glucuronide conjugates - was carried out. The analysis showed
404 significant increases in aronia-derived plasma (poly)phenols upon intervention.
405 Various studies demonstrated that anthocyanins are broken down in several key
406 intermediates, including hippuric acid, protocatechuic acid, and phenylproprionic acids

(38-41). This is in agreement with our results where hippuric acids, cinnamic acids and propionic acids were increased after consumption of both aronia treatments (**Supplementary figure 1**). Interestingly, 12-week baseline increases in total (poly)phenols was twice as high for the extract group (30 µM) as compared to the the whole fruit group (14 µM). Even though whole fruit treatment was low in (poly)phenols, the 14 µM increase in plasma polyphenols might explain the significant baseline FMD improvements of 0.8 % 12 weeks after consumption. In addition, twice the amount of phenylacetic acids were quantified in volunteers ingesting the aronia extract compared to aronia whole fruit, indicating a difference in absorption depending on the treatment given (**Supplementary Figure 2**). It must be considered that the (poly)phenol content in aronia whole fruit capsules might have been underestimated due to the presence of non-extractable (poly)phenols bound to fibers. Actually, it was previously shown that coarse milling, a milling process that creates 90 nm nanoparticles, could increase the total (poly)phenol content by 40 to 50 % (42). To further explore the relationship between aronia berry-derived (poly)phenols and vascular function, a correlation analysis was performed (**Table 4**). A total of 24 metabolites correlated significantly upon intake of aronia with the most abundant ones being hydroxycinnamic acids and benzoic acids (**Table 4**). Interestingly, no correlations were found after acute (2 h) or chronic (12 wks) intake of whole fruit capsules. Assessing the bioactivity of dietary (poly)phenols *in vivo* remains a challenge and is still largely unknown. It is believed that dietary (poly)phenols in the nM range could increase nitric oxide (NO) availability and therefore improve NO-dependent FMD responses (44, 45). Over half of the quantified plasma phenolic acids in the current study were previously identified as gut microbial breakdown products (46, 47) and other than improving vascular health, phenolic acids are also believed to modulate the gut microbiome (48). Research

suggests that changes in microbial communities could protect against diet-induced obesity and cardiometabolic diseases (49). Furthermore, several studies have attributed prebiotic effects to (poly)phenols as they would improve growth of *Lactobacilli* and *Bifidobacteria* in several *in vitro* models and clinical studies (50, 51). To explore whether plasma (poly)phenol metabolites could, in part, be responsible for the changes in gut microbial abundancies observed, a correlation analysis was performed (**Figure 4**). Even though, most of the correlations were not significant, clearly more significant associations were found after intake of aronia extract compared to whole fruit. These findings show, that after 12 weeks of intake, volunteers in the extract group showed more associations with modulated gut microbe genera compared to the whole fruit group. In addition, our data identified a significant increase in *Anaerostipes* abundance after aronia extract consumption (**Supplementary figures 3 and 4**). It is suggested that the *Anaerostipes* genus plays an important functional role in the gut ecosystem due to the ability to produce butyrate from lactate (52). Butyrate was associated with beneficial effects in various diseases such as genetic metabolic diseases, hypercholesterolemia, insulin resistance, and ischemic stroke and colon cancer (53). A notable limitation of this work is that the study population consisted of a group of healthy young men. Therefore, our findings cannot be directly extrapolated to all segments of the general population. The study also has notable strengths: an extensive metabolomics analysis of plasma (poly)phenols in tandem with the primary outcome made it possible to correlate plasma levels with FMD as well as with gut microbial genera. In conclusion, our present data indicates that consumption of dietary achievable amounts of *Aronia melanocarpa* by healthy individuals can lead to clinically relevant improvements in endothelial function after an overnight fast. Furthermore, we linked plasma phenolic metabolites to vascular benefits and

457 observed significant changes in gut microbial populations. Our results indicate that
458 consumption of aronia berry (poly)phenols as part of a balanced and healthy diet may
459 help to maintain cardiovascular health in young individuals at low risk of CVD.

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Figure Legends

Figure 1A. Aronia study flow.

Figure 1B. Study protocol of the randomised controlled study. FMD, flow-mediated dilation; PWV, pulse wave velocity; AIX, augmentation index.

Figure 2A. FMD change from baseline (CFB) 2h post-consumption of the control, aronia whole fruit or aronia extract capsules.

Figure 2B. FMD change from baseline (CFB) three months after daily consumption of the control, aronia whole fruit or aronia extract capsules.

Figure 2C. FMD change from baseline (CFB) at 2h post-consumption and after three months daily consumption of the control, aronia whole fruit or aronia extract capsules.

Figure 3. Plasma (poly)phenol metabolites after consumption of aronia berries.
Figure 3A. Total hippuric acids
Figure 3B. Total benzoic acids
Figure 3C. Total cinnamic acids
Figure 3D. Total phenylacetic acids
Figure 3E. Total benzaldehydes
Figure 3F. Total catechols
Figure 3G. Total pyrogallols
Figure 3H. Total flavanols
Figure 3I. Total propionic acids

Figure 4. Correlation heatmap of plasma metabolites and gut microbiome. **A.** Changes in metabolite concentrations versus changes in abundance of microbial genera upon 12-week consumption of aronia extract and control (n = 43). **B.** Changes in metabolite concentrations versus changes in abundance of microbial genera upon 12-week consumption of aronia whole fruit and control (n = 43). Correlations were

performed including controls in two independent analyses: aronia extract + control and aronia whole fruit + control. Values are represented as spearman rho, *p < 0.05.

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Tables

Table 1. (Poly)phenol content of intervention capsule

Per capsule (500 mg)	**Extract**		**Whole fruit**		**Control**	
	Mean	SD	Mean	SD	Mean	SD
Flavonols (mg)	**35**	**0.3**	**2.6**	**0.1**	**0**	**0**
Quercetin (µg)	1625	395	575	430	0	0
Quercetin-3-glucoside (µg)	8879	970	534	33	0	0
Quercetin-3-galactoside (µg)	7859	68	816	42	0	0
Quercetin-3-rhamnoside (µg)	9	1.5	0	0	0	0
Quercetin-3-rutinoside (µg)	15797	613	610	20	0	0
Kaempferol (µg)	18	19	27	30	0	0
Kaempferol-3-glucoside (µg)	229	21	0	0	0	0
Myricetin (µg)	6.5	7	2.5	2.5	0	0
Myricetin-3-glucoside (µg)	76	8.5	0	0	0	0
Isorhamnetin (µg)	71	24	32	18	0	0
Hesperetin (µg)	1.5	1.5	1	1.5	0	0
Naringenin (µg)	5.5	1	2	0.5	0	0
Hydroxycinnamic acids (mg)	**33**	**0.4**	**1.7**	**0**	**0**	**0**
Vanillic acid (µg)	0.5	0.5	0	0	0	0
p-coumaric (µg)	119	17	3.5	4	0	0
m-coumaric (µg)	28	12	1.5	1.5	0	0
o-coumaric (µg)	11	4	0.5	0.5	0	0
Caffeic acid (µg)	819	22	125	1.5	0	0
Dihydrocaffeic acid (µg)	25	11	2.5	6.5	0	0
Ferulic acid (µg)	84	35	6.5	4	0	0
Isoferulic acid (µg)	225	150	17	19	0	0
Neochlorogenic acid (µg)	8360	374	425	49	0	0
Chlorogenic acid (µg)	21341	1180	1116	50	0	0
Cryptochlorogenic (µg)	1746	17	30	2	0	0
Benzoic acids (mg)	**2.8**	**0**	**0.5**	**0**	**0**	**0**
Benzoic acid (µg)	295	23	16	17	0	0
Gallic acid (µg)	61	15	1	0.5	0	0
4-hydroxybenzoic acid (µg)	59	1.5	23	3	0	0
3-hydroxybenzoic acid (µg)	6.5	7	0.5	0.5	0	0
2-Hydroxybenzoic acid (µg)	25	28	5	5.5	0	0
Protocatechuic acid (µg)	2396	105	450	18	0	0
Phloretin (µg)	0.5	0.5	0	0	0	0
Epicatechin (µg)	101	5.5	0	0	0	0
Total phenolics (mg)	**70**	**1.5**	**4.8**	**0.6**	**0**	**0**
Anthocyanins (mg)	**29**	**-**	**3.6**	**-**	**0**	**0**
Proanthocyanidins (mg)	**16**	**3.2**	**3.3**	**0.9**	**0**	**0**
Total (poly)phenols (mg)	**115**	**4.7**	**12**	**1.4**	**0**	**0**

Table 2. Baseline characteristics of the population included in the study.

Population characteristics	All	Control	Whole fruit	Extract
		Mean ± SD		
Age (years)	24 ± 5.3	23 ± 4.4	24 ± 5.2	24 ± 6.3
Height (cm)	177 ± 7.2	176 ± 8.5	176 ± 5.9	178 ± 7.3
Weight (Kg)	71 ± 8.3	69 ± 6.8	70 ± 9.8	74 ± 7.5
BMI (kg/m^2)	23 ± 2.1	22 ± 1.6	23 ± 2.6	23 ± 1.9
PSBP (mmHg)	119 ± 10.6	118 ± 11	119 ± 8.4	119 ± 12.6
PDBP (mmHg)	68 ± 7.9	68 ± 6.2	69 ± 9.1	67 ± 8.2
CSBP (mmHg)	101 ± 7.9	100 ± 7	102 ± 8.3	101 ± 8.6
CDBP (mmHg)	70 ± 9.2	70 ± 7.4	71 ± 10.1	69 ± 9.8
HR (bpm)	62 ± 9.8	62 ± 8.9	63 ± 9.4	62 ± 11.4
PWV (m/s)	5.5 ± 1.1	5.1 ± 1	5.7 ± 1.2	5.6 ± 1.2
AIx (%)	-3.6 ± 10	-3.8 ± 10.7	-2.6 ± 9.4	-4.4 ± 10.3
Body fat (%)	15 ± 4.0	15 ± 3.4	14 ± 4.4	15 ± 3.9
BMR (Kcal)	1787 ± 188	1736 ± 191	1770 ± 192	1848 ± 171
PGLU (mmol/L)	5 ± 0.3	4.9 ± 0.3	5.0 ± 0.4	5 ± 0.3
PLT (10^9/L)	225 ± 38.8	228 ± 31.9	221 ± 38	225 ± 46.1
Urea (mmol/L)	5.8 ± 1.3	5.9 ± 1.5	5.5 ± 0.8	6.0 ± 1.5
Creatin (mmol/L)	80 ± 10.2	77 ± 9	77 ± 7.7	84 ± 12.2
ALP (IU/L)	66 ± 16.3	70 ± 18.4	63 ± 16.4	65 ± 14.3
AST (IU/L)	26 ± 12.6	23 ± 6.7	24 ± 7.7	30 ± 18.5
GGT (IU/L)	17 ± 14.4	17 ± 9.5	16 ± 11.3	19 ± 20.2
CHOL (mmol/L)	4.1 ± 0.7	4.1 ± 0.8	4 ± 0.6	4.3 ± 0.7
TRIG (mmol/L)	0.8 ± 0.4	0.8 ± 0.3	0.7 ± 0.3	1.0 ± 0.5
HDL (mmol/L)	0.4 ± 0.3	1.3 ± 0.2	1.5 ± 0.3	1.3 ± 0.2
LDL (mmol/L)	2.4 ± 0.6	2.4 ± 0.8	2.2 ± 0.5	2.5 ± 0.6
LDH (IU/L)	150 ± 22.9	147 ± 22.6	147 ± 27.6	156 ± 17
Smoking (%)	14	20	13	9

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Table 3. Plasma (poly)phenols correlation with FMD.

Δ FMD vs Δ plasma (poly)phenols	Aronia extract (2 h)	Aronia extract (12 wks)	Aronia extract (12 wks, 2 h)	Aronia whole fruit (12 wks, 2 h)
		Spearman ρ		
Total (poly)phenols	0.34			
2-Hydroxyhippuric acid	0.36			
Protocatechuic acid			0.41	
2-Hydroxybenzoic acid			0.30	
3-Hydroxybenzoic acid		0.30	0.41	0.38
4-Hydroxybenzoic acid	0.35			
Vanillic acid-4'-*O*-sulphate	0.33			
Isovanillic acid		0.31		
Gallic acid			0.45	
Dihydroferulic acid	0.58			
Dihydro isoferulic acid	0.41			
p-Coumaric acid			0.30	
o-Coumaric acid				0.36
Ferulic acid-4'-*O*-sulphate				0.32
Isoferulic acid-3'-*O*-β-*D*-glucuronide	0.39			
Dihydroferulic acid-4'-*O*-β-*D*-glucuronide	0.32			
Dihydro isoferulic acid-3'-*O*-β-*D*-glucuronide	0.37			
Dihydro isoferulic acid-3'-*O*-sulphate		0.35		
Phenylacetic acid	0.48		0.44	0.33
3,4-Dihydroxyphenylacetic acid				0.31
4-Hydroxybenzaldehyde			0.39	
Catechol-*O*-sulphate	0.44			
Kaempferol			0.42	
Quercetin-3'-*O*-β-*D*-glucuronide	0.40			
(4R)-5-(3'-Hydroxyphenyl)-γ-valerolactone-4'-*O*-sulfate	0.39			

Correlations between changes in plasma metabolite concentrations (with respect to baseline) and FMD changes (with respect to baseline). Correlations were performed by correlating control and aronia extract outcomes in one analysis and perform a second control and aronia whole fruit correlation in an independent analysis. This was repeated for data obtained from acute (2 h), chronic (12 wks) and acute on chronic (12 wks, 2 h). No correlations were found between aronia whole fruit and FMD on the acute and chronic level. Spearman was used for correlations of non-parametric data. All data represented had $p < 0.05$.

Supplementary material

Supplementary Figure 1. Plasma (poly)phenol concentrations. Figure 3A, 3B, 3C and 3D display the plasma concentration of 63 quantified (poly)phenols.

Supplementary Figure 2. The pie charts display the ratio of abundance (%) of (poly)phenol groups with respect to total (poly)phenols in plasma after substraction of baseline 12-weeks from the baseline values on day 1 in extract and whole fruit consumers.

Supplementary Figure 3. Changes in the gut microbiome associated to aronia treatments. Top 10 features from aronia extract (A), aronia whole fruit and control (C) data sets, as revealed by Random Forests. Red dots denote bacterial genera significantly discriminant of the final microbiome structure respect to the initial configuration for each treatment. Differences in the relative abundances of each relevant genera were investigated using Wilcoxon signed-rank tests and visualized by both dot plots and box plots. Paired samples from the same individual were connected by a black line.

Supplementary Figure 4. Significant increases in *Anaerostipes* abundancy upon intake of the aronia extract compared to control. Significance was tested using a paired t-test of the changes in microbial abundanties.

Figure 1

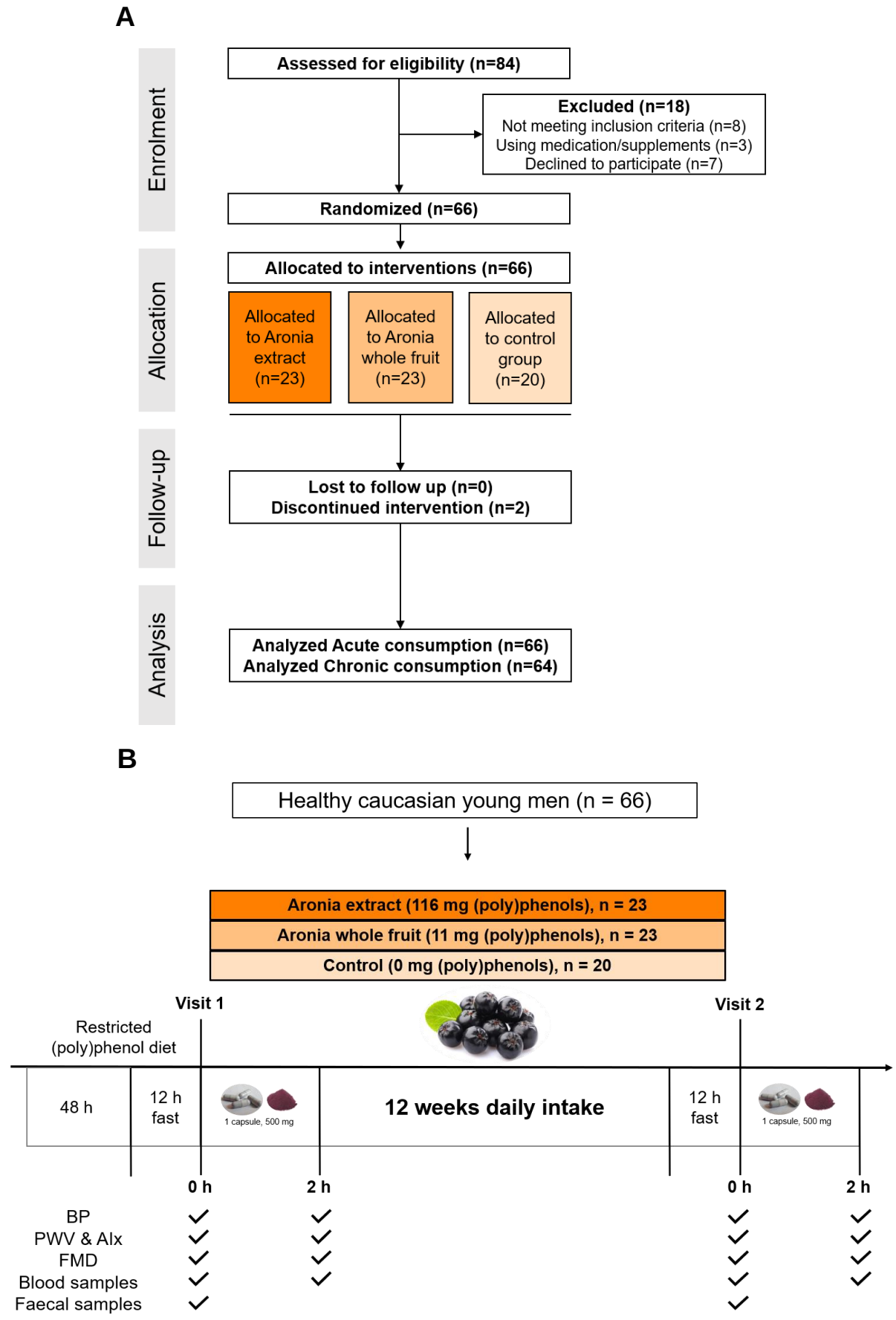

A
Enrolment
Assessed for eligibility (n=84)
Excluded (n=18)
Not meeting inclusion criteria (n=8)
Using medication/supplements (n=3)
Declined to participate (n=7)
Randomized (n=66)
Allocation
Allocated to interventions (n=66)
Allocated to Aronia extract (n=23)
Allocated to Aronia whole fruit (n=23)
Allocated to control group (n=20)
Follow-up
Lost to follow up (n=0)
Discontinued intervention (n=2)
Analysis
Analyzed Acute consumption (n=66)
Analyzed Chronic consumption (n=64)
B
Healthy caucasian young men (n = 66)
Aronia extract (116 mg (poly)phenols), n = 23
Aronia whole fruit (11 mg (poly)phenols), n = 23
Control (0 mg (poly)phenols), n = 20
Visit 1
Visit 2
Restricted (poly)phenol diet
48 h
12 h fast
1 capsule, 500 mg
12 weeks daily intake
12 h fast
1 capsule, 500 mg
0 h
2 h
0 h
2 h
BP
PWV & AIx
FMD
Blood samples
Faecal samples

Figure 2


A
p = 0.005
p = 0.036
% ΔFMD (12 wks)
1.5
1.0
0.5
0.0
-0.5
Control
Whole fruit
Extract
B
p = 0.003
p = 0.011
% ΔFMD (2 h)
2.0
1.5
1.0
0.5
0.0
Control
Whole fruit
Extract
C
p < 0.001
p = 0.005
% ΔFMD (12 wks, 2 h)
2.0
1.5
1.0
0.5
0.0
-0.5
Control
Whole fruit
Extract

Figure 3

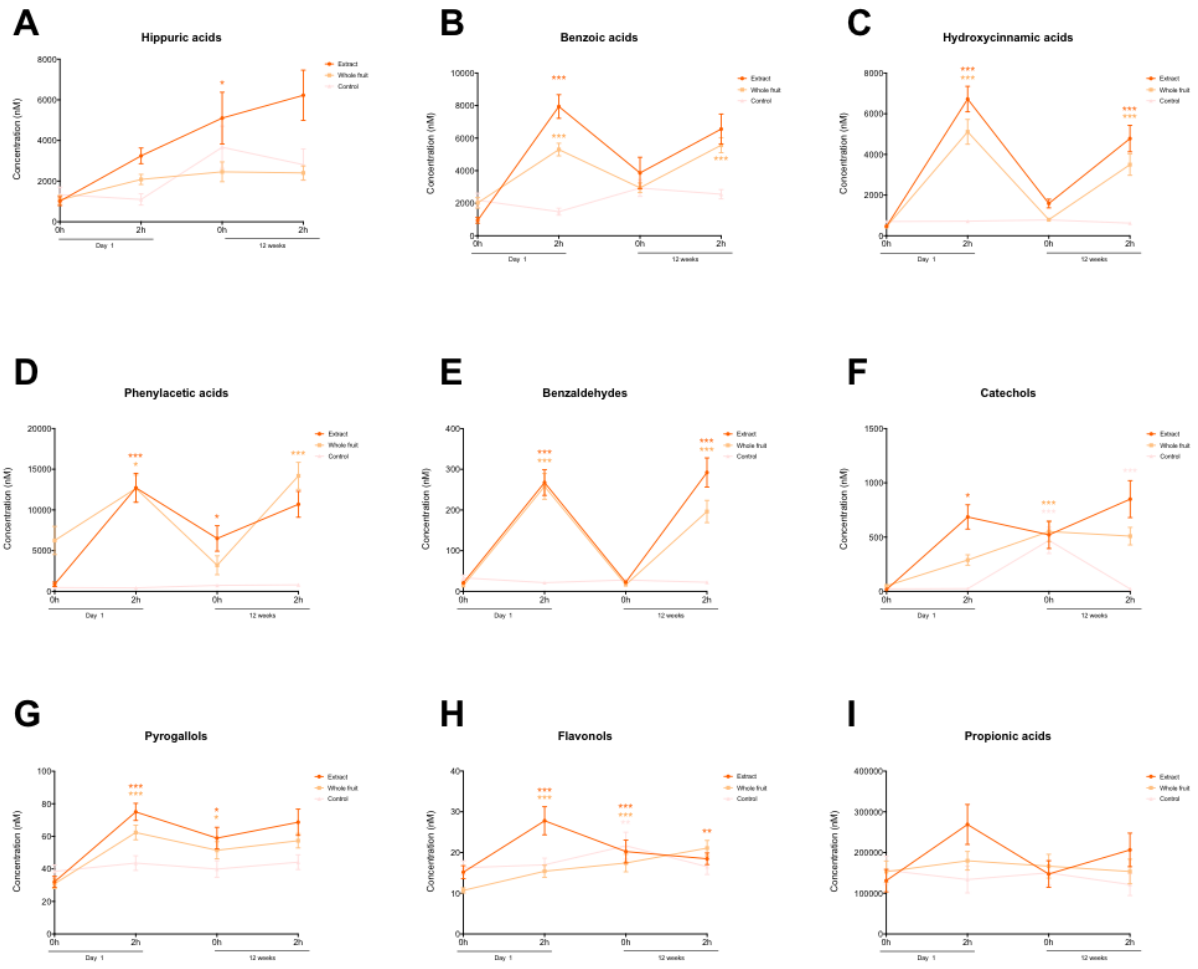
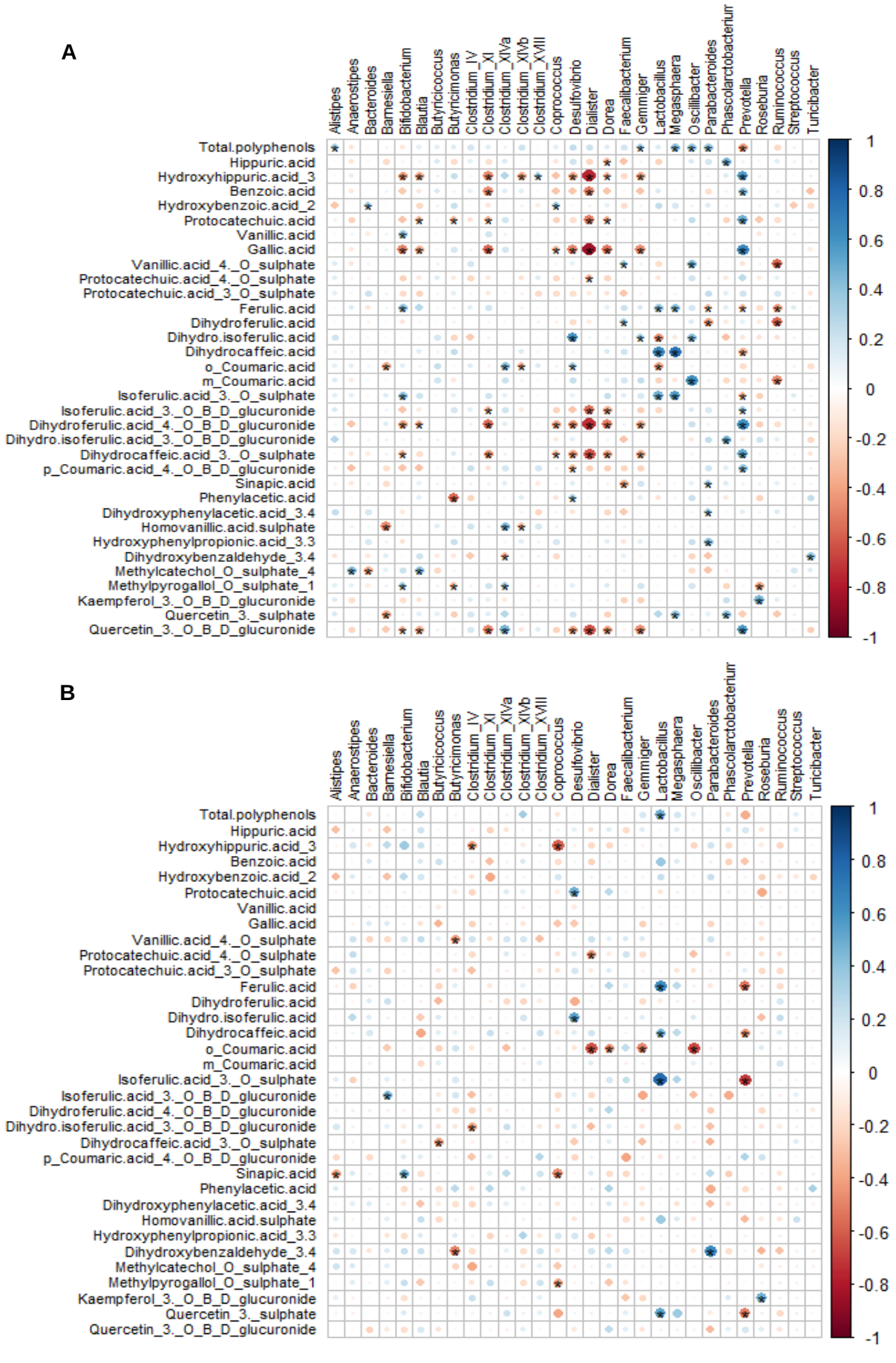
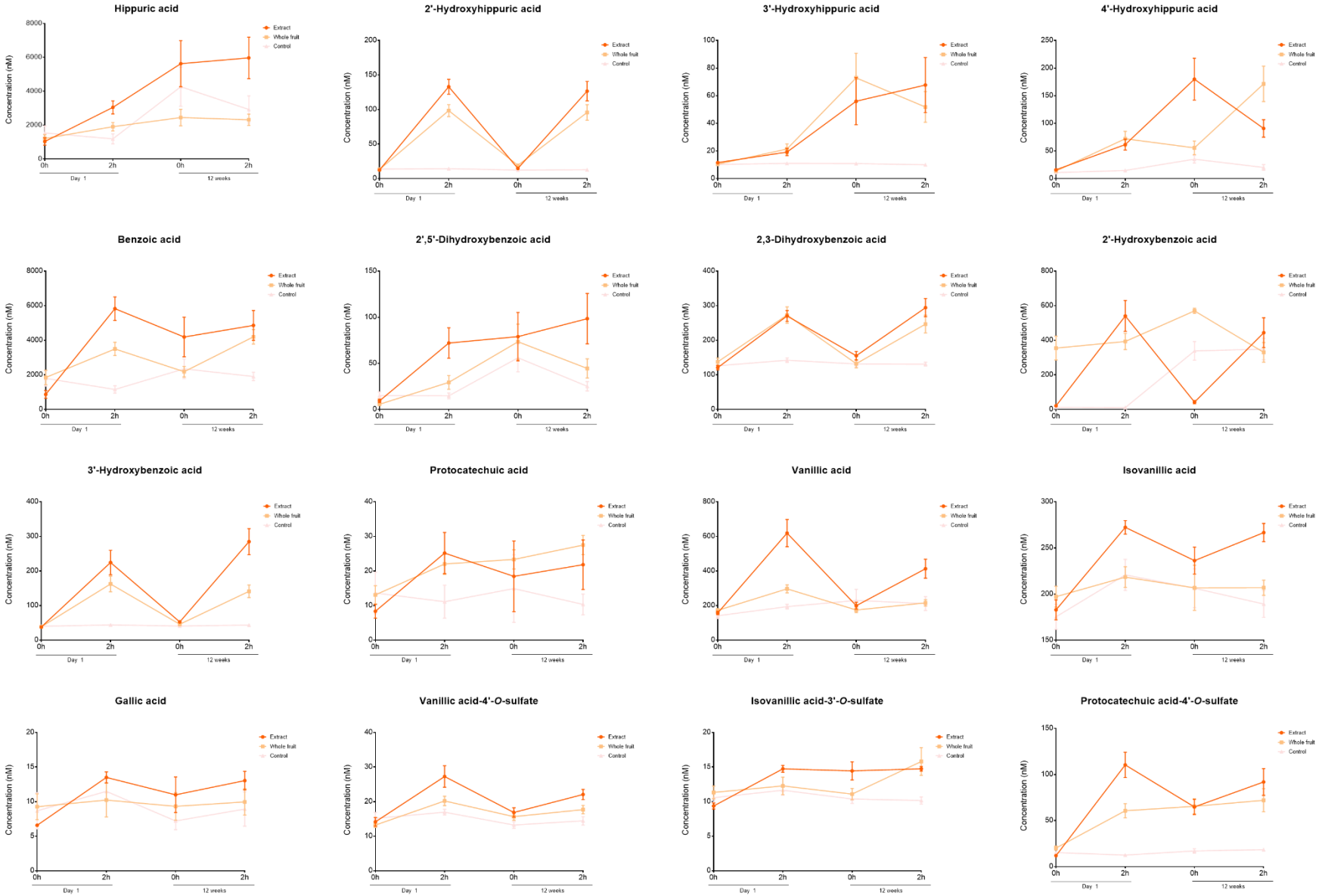

A
Hippuric acids
B
Benzoic acids
C
Hydroxycinnamic acids
D
Phenylacetic acids
E
Benzaldehydes
F
Catechols
G
Pyrogallols
H
Flavonols
I
Propionic acids
Concentration (nM)
Extract
Whole fruit
Control
0h
2h
Day 1
12 weeks

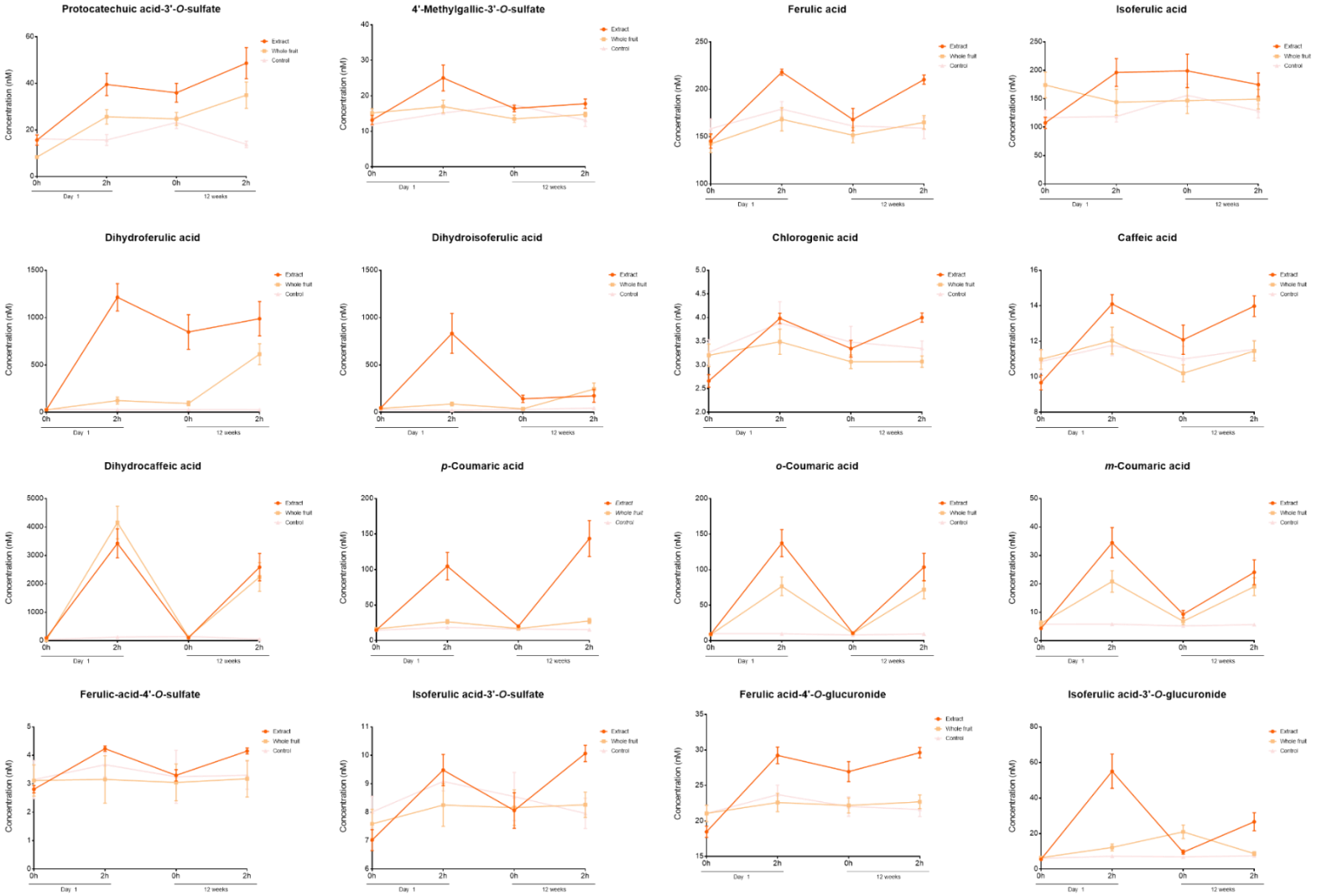
Figure 4


A
Alistipes
Anaerostipes
Bacteroides
Barnesiella
Bifidobacterium
Blautia
Butyricicoccus
Butyricimonas
Clostridium_IV
Clostridium_XI
Clostridium_XIVa
Clostridium_XIVb
Clostridium_XVIII
Coprococcus
Desulfovibrio
Dialister
Dorea
Faecalibacterium
Gemmiger
Lactobacillus
Megasphaera
Oscillibacter
Parabacteroides
Phascolarctobacterium
Prevotella
Roseburia
Ruminococcus
Streptococcus
Turicibacter
Total.polyphenols
Hippuric.acid
Hydroxyhippuric.acid_3
Benzoic.acid
Hydroxybenzoic.acid_2
Protocatechuic.acid
Vanillic.acid
Gallic.acid
Vanillic.acid_4._O_sulphate
Protocatechuic.acid_4._O_sulphate
Protocatechuic.acid_3_O_sulphate
Ferulic.acid
Dihydroferulic.acid
Dihydro.isoferulic.acid
Dihydrocaffeic.acid
o_Coumaric.acid
m_Coumaric.acid
Isoferulic.acid_3._O_sulphate
Isoferulic.acid_3._O_B_D_glucuronide
Dihydroferulic.acid_4._O_B_D_glucuronide
Dihydro.isoferulic.acid_3._O_B_D_glucuronide
Dihydrocaffeic.acid_3._O_sulphate
p_Coumaric.acid_4._O_B_D_glucuronide
Sinapic.acid
Phenylacetic.acid
Dihydroxyphenylacetic.acid_3.4
Homovanillic.acid.sulphate
Hydroxyphenylpropionic.acid_3.3
Dihydroxybenzaldehyde_3.4
Methylcatechol_O_sulphate_4
Methylpyrogallol_O_sulphate_1
Kaempferol_3._O_B_D_glucuronide
Quercetin_3._sulphate
Quercetin_3._O_B_D_glucuronide
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
B
Alistipes
Anaerostipes
Bacteroides
Barnesiella
Bifidobacterium
Blautia
Butyricicoccus
Butyricimonas
Clostridium_IV
Clostridium_XI
Clostridium_XIVa
Clostridium_XIVb
Clostridium_XVIII
Coprococcus
Desulfovibrio
Dialister
Dorea
Faecalibacterium
Gemmiger
Lactobacillus
Megasphaera
Oscillibacter
Parabacteroides
Phascolarctobacterium
Prevotella
Roseburia
Ruminococcus
Streptococcus
Turicibacter
Total.polyphenols
Hippuric.acid
Hydroxyhippuric.acid_3
Benzoic.acid
Hydroxybenzoic.acid_2
Protocatechuic.acid
Vanillic.acid
Gallic.acid
Vanillic.acid_4._O_sulphate
Protocatechuic.acid_4._O_sulphate
Protocatechuic.acid_3_O_sulphate
Ferulic.acid
Dihydroferulic.acid
Dihydro.isoferulic.acid
Dihydrocaffeic.acid
o_Coumaric.acid
m_Coumaric.acid
Isoferulic.acid_3._O_sulphate
Isoferulic.acid_3._O_B_D_glucuronide
Dihydroferulic.acid_4._O_B_D_glucuronide
Dihydro.isoferulic.acid_3._O_B_D_glucuronide
Dihydrocaffeic.acid_3._O_sulphate
p_Coumaric.acid_4._O_B_D_glucuronide
Sinapic.acid
Phenylacetic.acid
Dihydroxyphenylacetic.acid_3.4
Homovanillic.acid.sulphate
Hydroxyphenylpropionic.acid_3.3
Dihydroxybenzaldehyde_3.4
Methylcatechol_O_sulphate_4
Methylpyrogallol_O_sulphate_1
Kaempferol_3._O_B_D_glucuronide
Quercetin_3._sulphate
Quercetin_3._O_B_D_glucuronide
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1

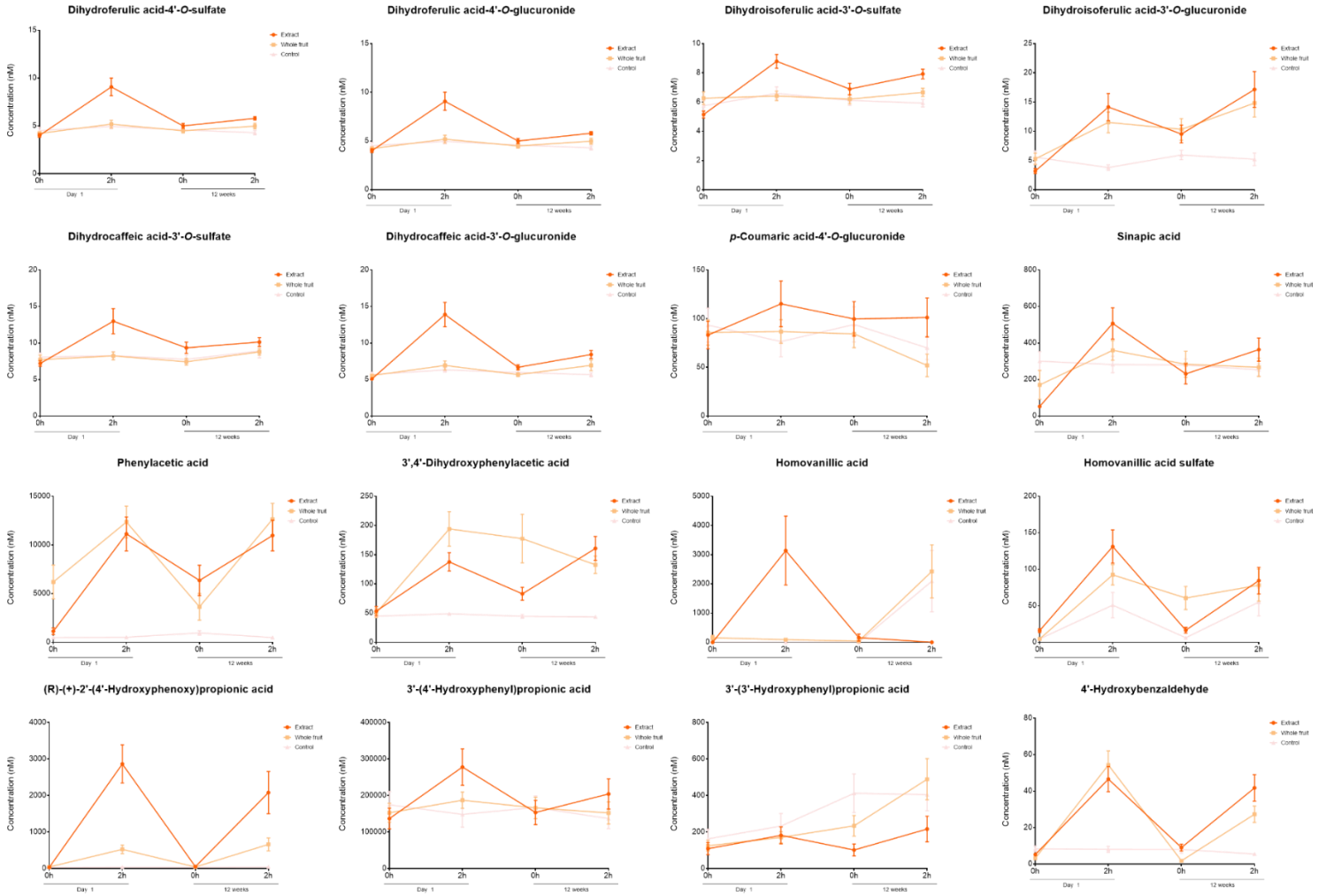
Supplementary figure 1A



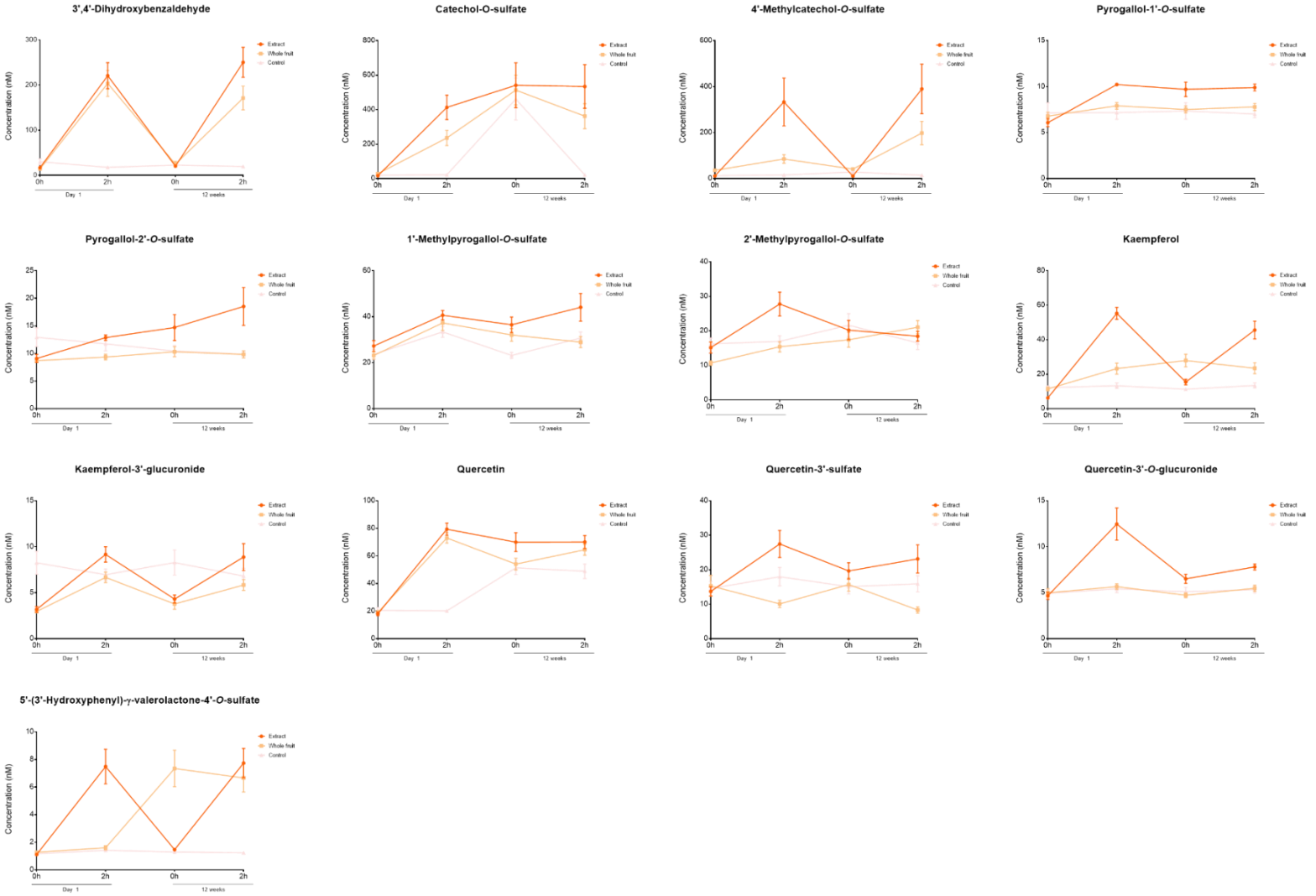
Supplementary figure 1B



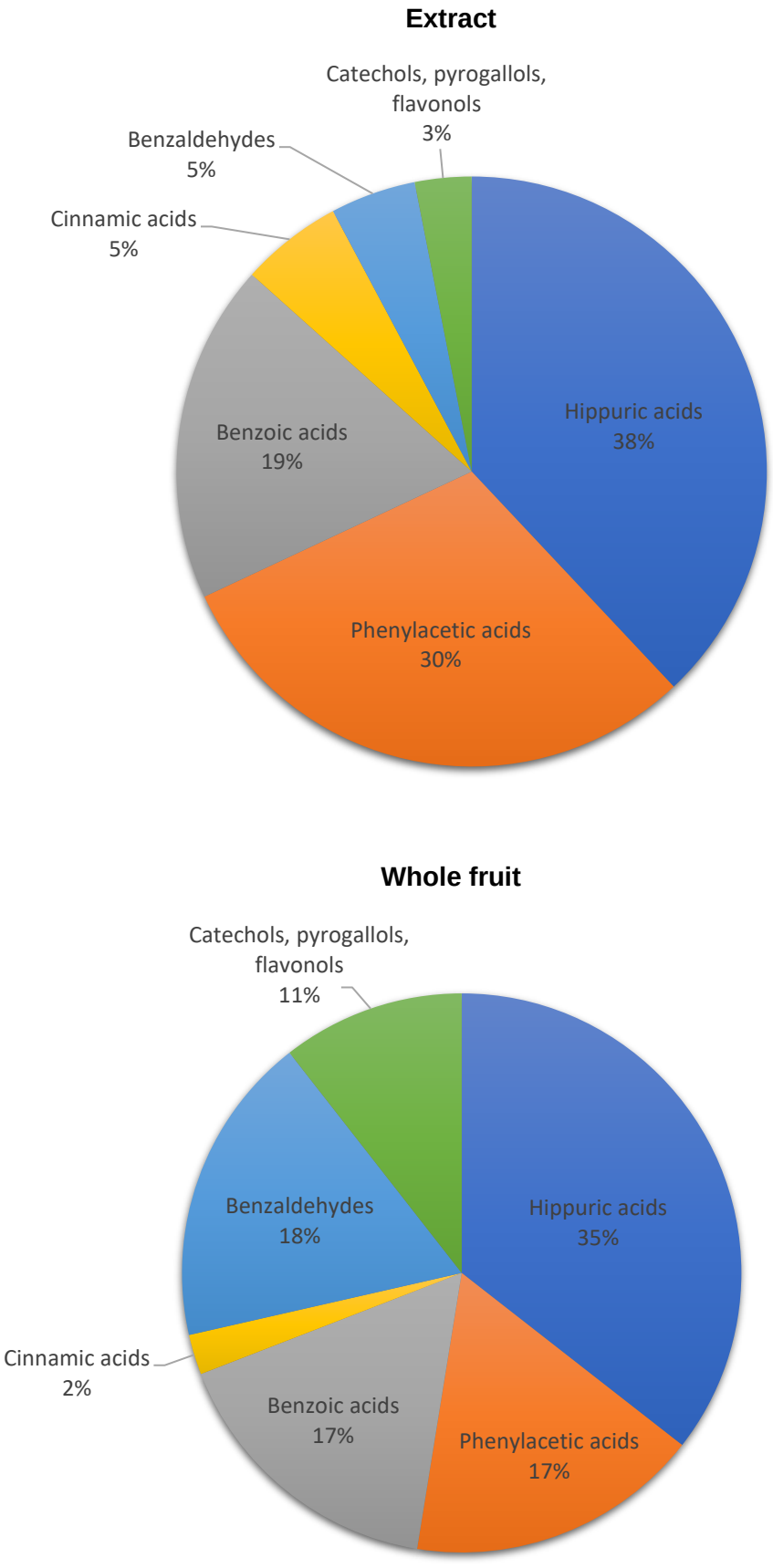
Supplementary figure 1C



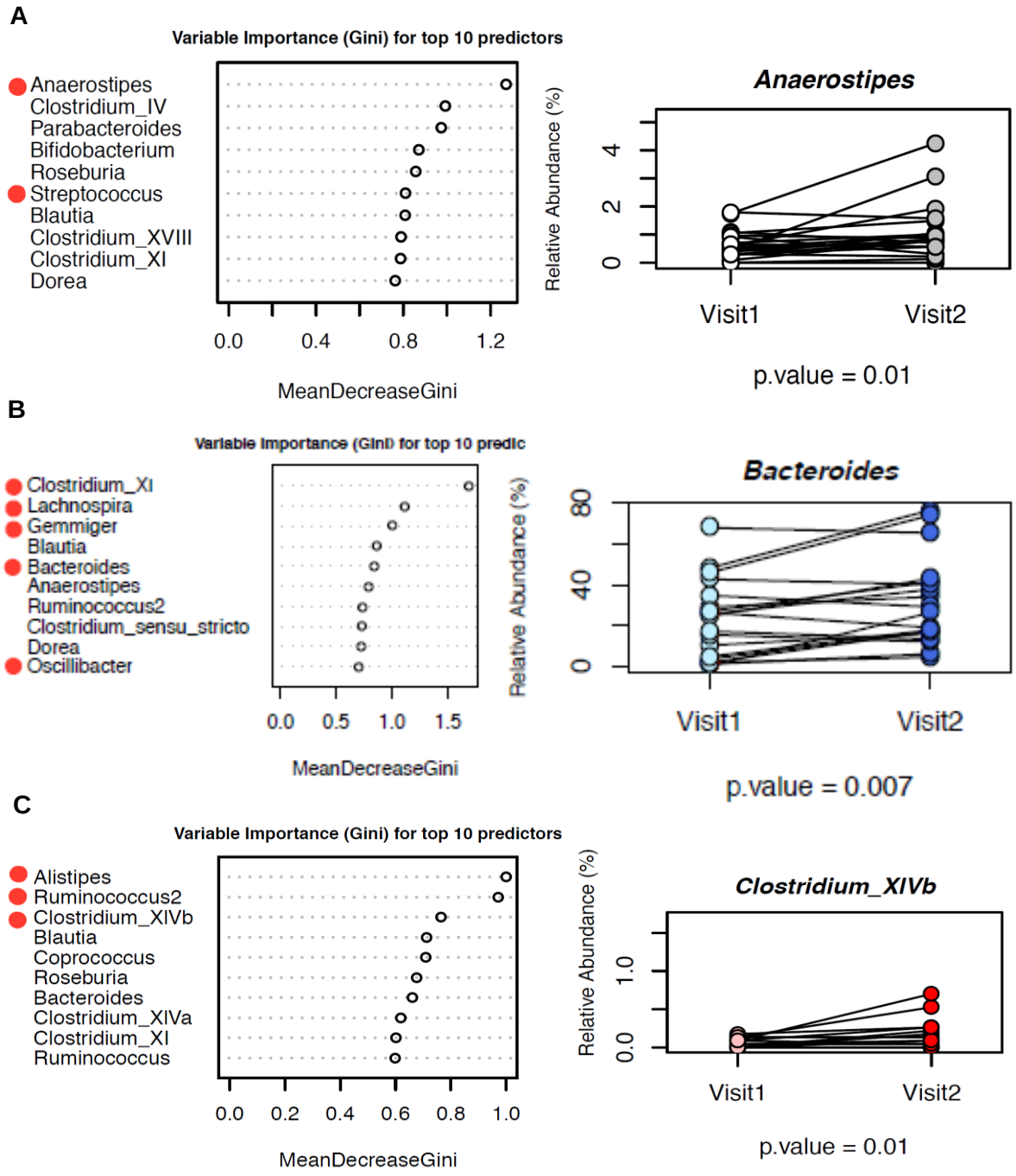
Supplementary figure 1D



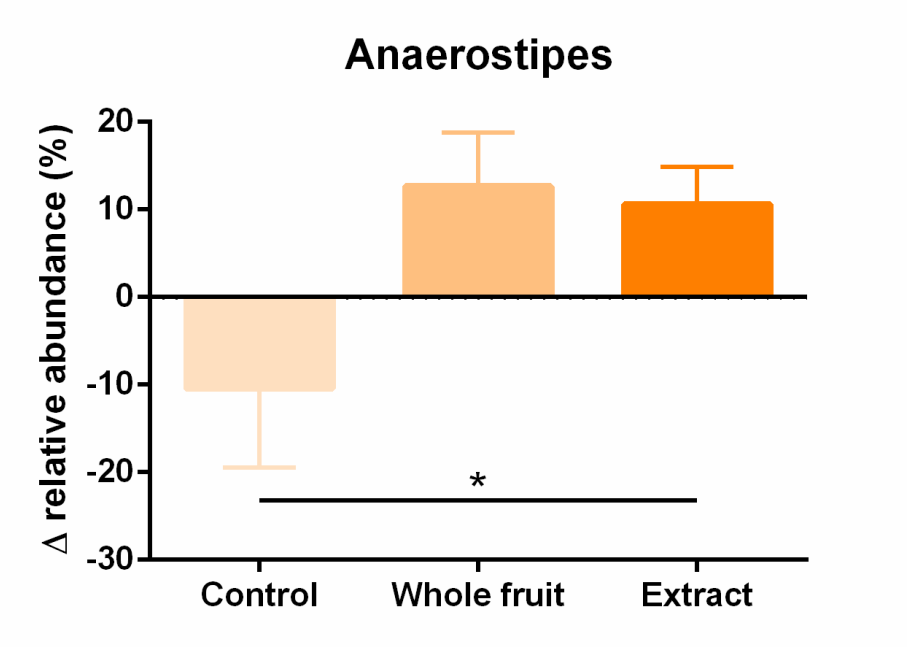
Supplementary Figure 2



Supplementary figure 3.



Supplementary figure 4.



Supplementary Table 1. Nutrient content of the three intervention capsules.

	Aronia whole fruit	Aronia extract	Control
Total fat (mg)	30	20	0.1
Protein (mg)	30	0.1	0.1
Total carbohydrates (mg)	480	530	550
Calories (cal)	2300	2300	2200
Dietary fibres, Total (mg)	260	120	20
Insoluble (mg)	30	0.1	0.1
Soluble (mg)	240	120	10
Vitamin C (mg)	70	0.1	20

Supplementary Table 2 - Safety

	Normal range	Control		Whole fruit		Extract		Δcontrol	P-value	Δfull spectrum	P-value	Δextract	P-value	Difference (Δwhole fruit - Δcontrol)	Difference (Δextract - Δcontrol)
		Visit 1	Visit 2	Visit 1	Visit 2	Visit 1	Visit 2								
Na (mmol/L)	**135-145**	140.9 ± 1.7	140.9 ± 1.5	140.8 ± 1.1	141.6 ± 1.1	141.5 ± 1.6	141.4 ± 1.7	-0.005263 (-1.14; 1.13)	0.9	0.836 (-0.22; 1.89)	0.003*	-0.06917 (-1.13; 0.99)	0.8	0.9163 (-0.28; 2.11)	-0.03828 (-1.23; 1.16)
Urea (mmol/L)	**3.4-7**	5.9 ± 1.5	5.8 ± 1.7	5.5 ± 0.8	5.4 ± 1.6	6 ± 1.5	5.2 ± 1.4	-0.04816 (-1.15; 1.06)	0.9	-0.08696 (-1.11; 0.93)	0.8	-0.7879 (-1.82; 0.24)	0.01*	0.00778 (-1.01; 1.03)	-0.6144 (-1.64; 0.41)
Total protein (g/L)	**60-80**	69.7 ± 4.2	68.6 ± 3.6	71.6 ± 3.3	68.9 ± 3.1	70.7 ± 3.5	69.3 ± 6.8	-1.068 (-4.39; 2.26)	0.2	-2.702 (-5.80; 0.39)	0.004*	-1.421 (-4.52; 1.68)	0.2	-1.517 (-4.86; 1.83)	-0.3349 (-3.68; 3.01)
Albumin (g/L)	**35-50**	43.7 ± 2.2	43.2 ± 1.6	45.1 ± 2.5	43.1 ± 1.6	43.8 ± 1.7	43.8 ± 2.7	-0.5421 (-2.18; 1.09)	0.2	-1.951 (-3.47; -0.43)**	0.0003*	-0.05336 (-1.57; 1.47)	0.8	-1.414 (-3.06; 0.23)	0.4952 (-1.15; 2.14)
Total bilirubin (umol/L)	**3-20**	13.3 ± 5.8	11.8 ± 3.9	15 ± 6.7	12.3 ± 6.7	11.3 ± 3.9	11.9 ± 4.9	-1.408 (-5.66; 2.85)	0.4	-2.682 (-6.64; 1.28)	0.007*	0.5593 (-3.40; 4.52)	0.7	-1.38 (-5.13; 2.37)	1.711 (-2.04; 5.46)
Alkaline phosphatase (IU/L)	**30-130**	69.5 ± 18.4	71.7 ± 21.3	62.6 ± 16.4	56.3 ± 16.4	65.4 ± 14.3	63.5 ± 14.3	2.237 (-10.86; 15.33)	0.6	-6.291 (-18.48; 5.90)	0.003*	-1.935 (-14.12; 10.25)	0.5	-6.904 (-14.06; 0.26)	-3.041 (-10.2; 4.12)
Mean corpuscular volume (fL)	**77-95**	91.3 ± 3.8	89.8 ± 5.4	92 ± 5.5	91.1 ± 5.5	92.7 ± 3.7	92.2 ± 3	-1.504 (-5.09; 2.08)	0.02*	-0.887 (-4.18; 2.41)	0.0006*	-0.5318 (-3.90; 2.84)	0.04*	0.5867 (-0.70; 1.88)	0.6451 (-0.67; 1.96)
Mean corpuscular hemoglobin concentration (g/dL)	**320-370**	329.2 ± 18.2	332.4 ± 8.4	326.5 ± 11.6	332 ± 9.3	327.9 ± 9.4	329.3 ± 10.8	3.271 (-5.79; 12.33)	0.5	5.478 (-2.86; 13.82)	0.004*	1.409 (-7.12; 9.94)	0.7	2.32 (-6.82; 11.46)	-2.301 (-11.64; 7.04)
Red cell distribution width (%)	**7.4-10.4**	13 ± 0.5	13.4 ± 0.5	13.2 ± 0.6	13.2 ± 0.7	13 ± 0.4	13.2 ± 0.4	0.4224 (-0.31; 1.15)	0.004*	0.2043 (-0.47; 0.88)	0.9	0.1636 (-0.52; 0.85)	0.1	-0.1957 (-0.62; 0.23)	-0.2619 (-0.70; 0.17)
Mean platelet volume (fL)	**11.0-15.0**	8.6 ± 0.8	9 ± 1.3	8.9 ± 0.8	9.1 ± 0.8	8.9 ± 0.9	9 ± 1	0.3387 (-0.08; 0.76)	0.03*	0.01304 (-0.37; 0.40)	0.04*	0.1773 (-0.22; 0.57)	0.3	-0.3343 (-0.64; 0.03)	-0.1426 (-0.45; 0.17)

Values are presented as mean ± SD

Supplementary Table 3. Average macro-, micronutrient and (poly)phenol intakes taken from 7-day diet diaries at baseline and during the 12-week intervention. Values are represented as mean ± SEM

	Control		Aronia whole fruit		Aronia extract	
	Visit 1	**Visit 2**	**Visit 1**	**Visit 2**	**Visit 1**	**Visit 2**
Energy (kcal)	2182 ± 422	2136 ± 569	2206 ± 459	2110 ± 482	2049 ± 579	1970 ± 463
Protein (g)	100 ± 24	100 ± 35	98 ± 32	101 ± 30	110 ± 32	106 ± 35
Carbohydrates (g)	232 ± 64	229 ± 65	232 ± 40	209 ± 45	216 ± 66	198 ± 51
Fibres (g)	83 ± 16	82 ± 28	88 ± 26	85 ± 29	78 ± 26	76 ± 24
Fat (g)	19 ± 5	21 ± 5	20 ± 5	20 ± 6	19 ± 5	19 ± 6
Vitamin A (mg)	5 ± 3	5 ± 3	5 ± 2	5 ± 3	6 ± 4	6 ± 5
Thiamin (mg)	13 ± 3	14 ± 5	12 ± 3	13 ± 4	13 ± 4	13 ± 4
Riboflavin (mg)	12 ± 4	13 ± 5	13 ± 6	13 ± 5	13 ± 3	12 ± 3
Niacin (mg)	347 ± 107	339 ± 113	334 ± 112	338 ± 90	360 ± 110	353 ± 108
Vitamin B6 (mg)	16 ± 5	16 ± 5	16 ± 5	16 ± 4	16 ± 5	15 ± 4
Folates (µg)	1729 ± 597	1842 ± 561	1597 ± 553	1772 ± 562	1592 ± 482	1693 ± 554
Vitamin B12 (µg)	37 ± 12	38 ± 22	37 ± 20	41 ± 24	41 ± 17	45 ± 24
Vitamin C (mg)	612 ± 543	743 ± 425	554 ± 362	672 ± 490	542 ± 362	577 ± 383
Vitamin D (µg)	20 ± 11	18 ± 14	24 ± 16	28 ± 22	27 ± 14	27 ± 13
Vitamin E (mg)	69 ± 22	76 ± 30	77 ± 24	74 ± 37	67 ± 31	70 ± 25
Sodium (g)	21 ± 7	19 ± 6	19 ± 5	19 ± 5	17 ± 6	17 ± 6
Potassium (g)	22 ± 47	22 ± 57	21 ± 6	22 ± 7	22 ± 5	21 ± 6
Calcium (g)	6 ± 2	6 ± 2	6 ± 2	6 ± 2	6 ± 2	6 ± 2
Magnesium (g)	2202 ± 499	2237 ± 547	2187 ± 758	2234 ± 671	2088 ± 479	2054 ± 599
Phosphorus (g)	10 ± 2	10 ± 3	10 ± 4	11 ± 3	11 ± 3	10 ± 3
Iron (mg)	91 ± 26	98 ± 26	95 ± 45	90 ± 21	86 ± 23	84 ± 23
Copper (mg)	9.4 ± 3	23 ± 54	10* ± 4	24* ± 52	8.7 ± 3	8.7 ± 3
Zinc (mg)	7* ± 18	8* ± 40	72 ± 25	79 ± 31	75 ± 26	74 ± 30
Chloride (g)	28 ± 9	25 ± 8	27 ± 7	27 ± 7	25 ± 8	24 ± 8
Selenium (µg)	408 ± 120	399 ± 211	445 ± 192	426 ± 208	467 ± 188	468 ± 171
Fluoride (mg)	7 ± 6	8.1 ± 5	7.4 ± 4	6.6 ± 5	6.5 ± 4	6.9 ± 4
Iodine (µg)	831 ± 310	839 ± 516	902 ± 685	923 ± 602	947 ± 361	962 ± 397
Total polyphenols (mg)	419 ± 52	553 ± 86	568 ± 92	584 ± 84	606 ± 76	465 ± 73
Anthocyanins (mg)	11 ± 4	19 ± 7	20 ± 5.5	24 ± 7.3	34 ± 8.7	31 ± 9.3
Flavan-3-ols (mg)	71 ± 17	139 ± 44	153 ± 40	163 ± 37	130* ± 20	74* ± 13
Proanthocyanidins (mg)	46 ± 12	56 ± 11	52 ± 10	70 ± 13	78 ± 16	87 ± 24
Flavonols (mg)	37 ± 4.2	39 ± 1	45 ± 9	44 ± 0.7	42 ± 6.1	36 ± 0.7
Flavones (mg)	3.8 ± 0.8	5 ± 42	3.2 ± 0.7	3.4 ± 34	3 ± 0.6	3.6 ± 35
Phenolic acids (mg)	157 ± 36	192 ± 5	180 ± 36	156 ± 5.2	209 ± 45	149 ± 6.7
Stilbenes (mg)	0.1 ± 0.05	0.2 ± 0	0.2 ± 0.1	0.3 ± 0.2	0.1 ± 0.02	0.1 ± 0.03
Other[1] (mg)	93 ± 5.9	103 ± 4	115 ± 11	123 ± 14	102 ± 6.2	84 ± 8.7

**significant difference (Δvisit 2-visit 1) at p<0.05*

[1]Others: *Chalcones, Dihydrochalcones, Isoflavonoids, Lignans, Hydrobenzoic acids, Hydroxycinnamic acids, Hydroxyphenylacetic acids, Hydroxphenylpropanoic acids, Resveratrol, Other.*

Supplementary Table 3. *Effects of Aronia berry after 12-week consumption on vascular parameters, blood pressure and heart rate*

	Control				Aronia whole fruit				Aronia extract				Difference (Δ Aronia whole fruit - Δ Control)						Difference (Δ Aronia extract - Δ Control)					
													2 h		12 weeks		12 weeks, 2 h		2 h		12 wks		12 wks, 2 h	
	Baseline	2 h	12 wks	12 wks, 2 h	Baseline	2 h	12 wks	12 wks, 2 h	Baseline	2 h	12 wks	12 wks, 2 h	Mean value	95% CI	Mean value	95% CI	Mean value	95% CI	Mean value	95% CI	Mean value	95% CI	Mean value	95% CI
FMD (%)	6.4 ± 1.5	6.7 ± 1.7	6.2 ± 1.2	6.0 ± 1.4	6.1 ± 1.0	6.7 ± 1.4	7.3 ± 1.8	7.8 ± 1.8	5.9 ± 1.3	7.4 ± 1.6	6.9 ± 1.7	8.5 ± 1.9	0.07598	-0.62; 0.77	0.9273	0.13; 1.72*	0.6233	-0.23; 1.47	1.075	0.37; 1.78**	1.167	0.36; 1.97**	1.482	0.62; 2.34***
Blood flow velocity (cm/sec)	120 ± 29	106 ± 25	131 ± 27	107 ± 18	123 ± 24	111 ± 22	131 ± 23	113 ± 19	124 ± 22	106 ± 21	129 ± 20	110 ± 22	-5.266	-21.68; 11.15	-6.771	-24.95; 11.4	5.624	-5.22; 16.47	0.578	-16.01; 17.16	-3.862	-22.22; 14.5	6.381	-4.58; 17.34
PWV (m/s)	5 ± 1	5 ± 1	6 ± 1	6 ± 1	6 ± 1	6 ± 1	6 ± 1	6 ± 1	6 ± 1	5 ± 1	6 ± 1	6 ± 1	-0.5437	-1.22; 0.13	-0.1609	-0.83; 0.51	-0.2645	-0.93; 0.40	0.01914	-0.66; 0.70	0.1	-0.57; 0.77	-0.2847	-0.96; 0.39
AIx (%)	-4 ± 11	-11 ± 14	-2 ± 11	-6 ± 9	-4 ± 10	-13 ± 12	-3 ± 8	-9 ± 10	-3 ± 9	-9 ± 11	0 ± 10	-6 ± 12	0.913	-7.17; 9.00	1.405	-6.48; 9.29	-2.034	-7.10; 3.03	-2.545	-10.71; 5.62	-0.3756	-8.34; 7.59	-2.266	-7.38; 2.85
PSBP (mmHg)	118 ± 11	116 ± 10	119 ± 9	119 ± 14	119 ± 13	119 ± 12	118 ± 10	119 ± 10	119 ± 8	116 ± 7	119 ± 9	118 ± 9	0.6579	-5.58; 6.90	-0.5995	-6.78; 5.58	1.132	-3.98; 6.24	3.522	-2.78; 9.82	-0.4019	-6.65; 5.84	2.813	-2.35; 7.98
PDBP (mmHg)	68 ± 6	69 ± 8	71 ± 7	71 ± 7	67 ± 8	67 ± 9	70 ± 6	69 ± 7	68 ± 6	69 ± 8	71 ± 7	71 ± 7	-1.033	-5.63; 3.56	-1.815	-7.33; 3.70	0.3627	-4.27; 5.00	-0.5431	-5.19; 4.10	0.7931	-4.78; 6.37	-0.03947	-4.72; 4.64
CSBP (mmHg)	100 ± 7	99 ± 8	103 ± 7	99 ± 7	101 ± 9	100 ± 10	102 ± 8	100 ± 7	102 ± 8	99 ± 9	102 ± 9	101 ± 8	4.634	-5.22; 14.48	-1.776	-7.33; 3.78	2.719	-2.68; 8.11	5.94	-4.01; 15.89	-1.074	-6.69; 4.54	2.222	-3.23; 7.67
CDBP (mmHg)	70 ± 7	70 ± 6	72 ± 7	72 ± 7	69 ± 10	68 ± 9	72 ± 7	70 ± 7	71 ± 10	72 ± 10	71 ± 8	71 ± 7	4.668	-3.04; 12.37	-2.407	-9.4; 4.59	0.4714	-4.47; 5.41	2.751	-5.03; 10.53	1.249	-5.82; 8.31	-0.8804	-5.87; 4.11
Heart rate (bpm)	62 ± 9	55 ± 7	62 ± 9	56 ± 7	62 ± 11	57 ± 11	66 ± 12	60 ± 12	63 ± 9	55 ± 9	64 ± 11	56 ± 9	2.309	-4.25; 8.87	0.5286	-6.95; 8.01	-2.391	-7.09; 2.31	4.935	-1.69; 11.56	4.153	-3.40; 11.71	-0.2273	-4.98; 4.52

FMD, flow mediated dilation; PWV, pulse wave velocity; AIx, augmentation index; PSBP, peripheral systolic blood pressure; PDBP, peripheral diastolic blood pressure; CDBP, central systolic blood pressure; CDBP, central diastolic blood pressure

Differences are calculated from an unpaired t test comparing ARONIA WHOLE FRUIT and ARONIA EXTRACT with CONTROL changes from baseline

* p<0.05, ** p<0.01, *** p<0.001. Values are represented as mean ± SD

Supplementary Table 4 Effects of Aronia berries after 12-week consumption on blood lipids, fasting glucose and body composition

	Control		Aronia whole fruit		Aronia extract		Difference (Δ Aronia whole fruit - Δ Control)		Difference (Δ Aronia extract - Δ Control)	
	Visit 1	Visit 2	Visit 1	Visit 2	Visit 1	Visit 2				
Total cholesterol (mmol/L)	4.1 ± 0.8	4.3 ± 0.9	4.0 ± 0.6	3.9 ± 0.5	4.3 ± 0.7	4.2 ± 0.9	-0.2771	-0.69; 0.14	-0.1593	-0.58; 0.26
HDL-cholesterol (mmol/L)	1.3 ± 0.2	1.3 ± 0.3	1.5 ± 0.3	1.4 ± 0.3	1.3 ± 0.2	1.3 ± 0.2	-0.0754	-0.20; 0.05	-0.04833	-0.17; 0.08
LDL-cholesterol (mmol/L)	2.4 ± 0.8	2.6 ± 0.8	2.2 ± 0.5	2.1 ± 0.5	2.5 ± 0.6	2.6 ± 0.7	-0.1618	-0.50; 0.18	-0.0689	-0.41; 0.27
Triglycerides (mmol/L)	0.8 ± 0.3	0.8 ± 0.3	0.7 ± 0.3	0.8 ± 0.5	1.0 ± 0.5	0.9 ± 0.4	0.01533	-0.25; 0.28	-0.07775	-0.35; 0.19
Fasting glucose (mmol/L)	4.9 ± 0.3	5.0 ± 0.3	5.0 ± 0.4	5.2 ± 0.4	5.0 ± 0.3	5.2 ± 0.4	-0.1318	-0.81; 0.55	0.1057	-0.58; 0.79
Body weight (Kg)	69.1 ± 6.8	70.1 ± 7	70.1 ± 9.8	69.8 ± 9	73.9 ± 7.5	73.8 ± 7.7	-1.215	-2.65; 0.22	-0.9005	-2.35; 0.55
Body fat (%)	15.4 ± 3.4	15.8 ± 4	14.2 ± 4.4	14.1 ± 3.7	15.4 ± 3.9	14.7 ± 3.3	-0.4828	-2.05; 1.08	-0.7036	-2.28; 0.88
BMR (Kcal)	1736 ± 191	1752 ± 195	1770 ± 192	1766 ± 180	1848 ± 171	1855 ± 180	-19.25	-50.26; 12.05	-11.89	-43.52; 19.73

BMR, basal metabolic rate.